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In vitro evaluation of antifungal activity of soybean (*Glycine max*) seed coat proteins

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ABSTRACT

Proteins in the soybean seed coat have previously been characterized; however, the function of these proteins is unknown. We show that a soybean seed coat protein fraction was able to inhibit the growth of *Fusarium lateritium* and *Fusarium oxysporum* phytopathogenic fungi. The antifungal fraction isolated by DEAE-Sepharose chromatography revealed the presence of peroxidase, vicilin and a 24 kDa protein homologous to acid phosphatases. Germination experiments revealed that both acid phosphatase and peroxidase were exuded during seed imbibition. We suggest that the set of seed coat antifungal proteins may help protect seeds from colonization by phytopathogenic fungi.

1. Introduction

The seed coat is an important tissue for the regulation of imbibition and maintenance of the integrity of the seed (Bewley and Black, 1994) and is also the first seed barrier encountered by pests and pathogens (Moise et al., 2005). However, this tissue has been exclusively considered as a physical barrier and the involvement of seed coat molecules in this defensive role has not been considered. Seed cotyledons contain an array of proteins that may be involved in the protection of quiescent seeds against fungi. A 2S albumin-homologous protein from passion fruit seeds inhibited growth of the phytopathogenic fungi *Fusarium oxysporum* Schltdl. and *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, and the yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Agizzio et al., 2003). Chitinases and β-1, 3-glucanases from seeds of *Vigna unguiculata* (L.) Walp. seeds inhibited growth of *C. lindemuthianum* and *Colletotrichum musae* (Berk. & M.A. Curtis) Arx, (Gomes et al., 1996). Vicilins (7S storage proteins) from seeds of *V. unguiculata* were also able to inhibit fungal development (Gomes et al., 1997). Further reports have shown the presence of vicilin-like 7S storage proteins, normally thought to be expressed only in the embryo, in the seed coat of *Canavalia ensiformis* (L.) DC (Oliveira et al., 1999), *Phaseolus lunatus* L. (Moraes et al., 2000) and *Phaseolus vulgaris* L. (Silva et al., 2004). All these authors suggest that storage proteins play an important role in seed defense mechanisms against pests and pathogens. Soybean seed coat proteins have previously been identified, among these a 41 kDa peroxidase enzyme (Buttery and Buzzel, 1968; Gijzen, 1997), a 32 kDa class I chitinase (Gijzen et al., 2001) and a 21 kDa trypsin inhibitor (Kunitz, 1945; Koide and Ikenaka, 1973) and an 8 kDa hydrophobic protein (Gijzen et al., 1999). However, the functions of these proteins in this tissue are not known. The objective of our study is to investigate the presence of proteins with antifungal activity in the seed coat of *Glycine max* (L.) Merrill, which is a highly valuable edible crop throughout most of North and South America.

2. Materials and methods

2.1. Glycine max seeds and preparation of fungal inoculum

Soybean seeds were commercially acquired from local markets (Campos dos Goytacazes, RJ, Brazil) and stored at 4 °C prior to use. Fungal isolates utilized were *Fusarium lateritium* Nees and *F. oxysporum*, which were kindly supplied by CNPAF/EMBRAPA, Goiânia, Goiás, Brazil. The fungi were maintained on Sabouraud agar (1% peptone, 2% glucose and 1.7% agar–agar). For the preparation of *F. oxysporum* and *F. lateritium* conidia, the fungal cultures were transferred to Petri dishes containing Sabouraud agar, and allowed to grow for 12 days. After this period, 0.15 M NaCl solution (10 mL) was added to the dishes and these were

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gently agitated for 1 min for spore liberation with the help of a Drigalski loop. Spores were quantified in a Neubauer chamber for appropriate dilutions.

2.2. Isolation of the antifungal fraction

Seed coats were separated from cotyledons and ground to a fine powder; proteins were extracted (1:10 flour:buffer ratio) with 100 mM phosphate buffer with 500 mM NaCl, pH 7.6 for 3 h at 4 °C and centrifuged at 10,000 × *g* for 30 min. The supernatant obtained was treated with ammonium sulphate at 90% saturation for 24 h at 4 °C and centrifuged at 10,000 × *g* for 30 min at 4 °C. The precipitated fraction was dialysed against water for 24 h at 4 °C using dialysis tubing of molecular weight cut-off of 7 kDa. After dialysis, the precipitated fraction (ammonium sulphate precipitate fraction) and low molecular weight fraction (LMW) containing the molecules excluded during the dialysis were recovered by freeze-drying. The resulting ammonium sulphate precipitate fraction was fractionated using DEAE-Sepharose ion exchange chromatography. The sample was applied to a 14 × 2.5 cm column equilibrated with 50 mM potassium phosphate buffer, pH 7.6. After elution of the non-retained fraction (DI), adsorbed proteins were sequentially desorbed by 0.25 and 0.5 M NaCl (DII and DIII fractions, respectively).

2.3. Effects of seed coat fractions on fungal growth

To assay the effects of different chromatographic fractions (DI, DII and DIII) and LMW fraction on fungal growth, spores (1×10^4 mL^{-1} in 0.15 M NaCl solution) were incubated at 28 °C in microplates containing 100 μL of Sabouraud broth (6 g/100 mL) in the presence of 100 μL of each chromatographic fraction (400 μg P/mL) or 100 μL of LMW fraction (10 mg/mL) in 0.1 M sodium acetate buffer, pH 5.0. Optical readings at 600 nm were taken at zero time and at 6 h intervals for the following 48 h (Broekaert et al., 1990). Cell growth without addition of fractions was also determined. Experiments were run in triplicate and values obtained were used to calculate averages and standard deviations. The results were analysed through Student's *t*-test and significant differences were determined at $P<0.05$ (Bridge and Sawilowsky, 1999).

2.4. Protein characterization

Protein concentrations in the seed coat fractions were determined by the Bradford method (Bradford, 1976). The protein profiles were analysed by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970) and a 15% non-denaturing gel as described by Davis (1964). Electrophoresis was run at 15 mA and proteins (10 μg) were stained by 0.05% Coomassie Blue and destained in acetic acid (10%). DIII fraction proteins separated by SDS–PAGE were blotted on a PVDF membrane and the N-terminal amino acid sequence of a 24 kDa protein band was determined on a Shimadzu PPSQ-10 Automated Protein Sequencer performing the Edman degradation (Edman, 1950). PTH-amino acids were detected at 269 nm after separation on a reverse-phase C18 column under isocratic conditions.

2.5. Vicilin detection

DIII fraction proteins separated by SDS–PAGE were blotted on a nitrocellulose membrane and submitted to Western blotting detection (Towbin et al., 1979) using a primary antibody (anti-*V. unguiculata* cv. EPACE 10 vicilin antibody produced in rabbit) diluted at 1:2000 and a secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, Sigma) diluted at 1:2000. Immune reactions were developed using a BCIP/NBT tablet (Sigma) dissolved in 10 mL of distilled water for 10 min.

The levels of vicilin-like proteins were measured by the enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlman, 1971) using an anti-*V. unguiculata* cv. EPACE 10 vicilin primary antibody produced in rabbit (diluted 1:2000) and an alkaline phosphatase-conjugated anti-rabbit IgG second antibody (diluted 1:2000). *Vigna unguiculata* cv. EPACE 10 cotyledon vicilins, in concentrations of 5–0.0024 μg/100 μL, were used as standard. Alkaline phosphatase activity was revealed by adding 50 μL of a development solution (5 mg 4-nitrophenyl phosphate-*p*-NPP) in 5 mL of 100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.2) and the reaction was stopped by the addition of 50 μL 3 M NaOH. Absorbance was read at 410 nm. ELISA experiments were run in triplicate.

2.6. Enzyme detection

The presence of chitinase in the DIII fraction was determined by Western blotting (Towbin et al., 1979), using an anti-*Adenanthera pavonina* L. cotyledon chitinase antibody produced in rabbit, diluted at 1:1000, and a secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, Sigma) diluted at 1:2000. *Adenanthera pavonina* cotyledon chitinase was used as a positive control.

Acid phosphatase activity was measured according to the method of Ferreira et al. (1998), using *p*-nitrophenylphosphate (*p*-NPP) as substrate and 5 μg of the proteins from the DIII fraction. For determination of the optimal enzyme temperature, the reaction mixture was incubated from 20 to 60 °C for 25 min. Optimal time was determined by incubation of the reaction mixture for periods from 0 to 35 min at 37 °C. The optimal pH of the enzyme was analysed by performing the reactions at pH 2.0–9.0 using 100 mM glycine (pH 2.0, 3.0 and 9.0), 100 mM sodium acetate (4.0 and 5.0) and 150 mM Tris (6.0, 7.0 and 8.0). To determine the thermal stability of the enzyme, the fraction was preincubated at 30, 40, 50 and 60 °C for 10 min with further addition of *p*-NPP solution. The reaction was stopped by adding 1 mL of 1 M NaOH and the absorbance was read at 405 nm. One unit of activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μmol of *p*-NPP per minute.

Acid phosphatase activity was also detected on a 15% non-denaturing gel (Davis, 1964). The gel was washed with 150 mM Tris–HCl, pH 7.6 and incubated with 150 mM Tris–HCl, pH 7.6, 40 mM $CaCl_2$ and 10 mM *p*-NPP for 16 h. After this time, the gel was incubated with 1 M NaOH and calcium phosphate precipitation on the protein band was observed.

Total peroxidase activity was assayed as described by Hammerschmidt et al. (1982). The reaction mixture (1 mL) consisted of 0.25% (v/v) guaiacol in 10 mM sodium phosphate buffer, pH 6.0, containing 10 mM hydrogen peroxide. The tested sample (8.4 μL) was added to initiate the reaction, which was measured spectrophotometrically at 470 nm after 10 min. One unit of peroxidase activity was defined as the amount of enzyme that catalyses an absorbance increase of 0.01 per minute at 470 nm. Peroxidase activity was also detected using diaminobenzidine (DAB) as substrate on a 15% non-denaturing gel (Davis, 1964) and separated proteins were transferred to a nitrocellulose membrane. The membrane was rinsed in PBS (20 mM Na_2PO_4, 140 mM NaCl, pH 7.2), and incubated with the development solution (5 mg DAB, 100 μL of 2 M Tris, pH 7.5, 300 μL 0.1 M imidazole, 4.9 mL water, 5 μL H_2O_2) for 15 min.

Trypsin inhibitor activity was determined following the methodology described by Xavier-Filho and Coelho (1980) using BAPNA as substrate at a final concentration of 1 mM at 1% (v/v) of

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dimethyl sulfoxide (DMSO) in a final volume of 1.5 mL. Trypsin (20 μg) was dissolved in 500 μL of 50 mM Tris, 0.02 M $CaCl_2$, pH 8.2 and thermoequilibrated at 37 °C for 5 min before the addition of 300 μL of substrate to start the reaction which was allowed to proceed for 15 min at 37 °C. The reaction was stopped by the addition of 700 μL of 30% acetic acid. The absorbance was read at 410 nm. Trypsin inhibitory activity was determined by measuring the remaining activity of trypsin towards the substrate after pre incubation at 37 °C for 5 min with 50 μL of DIII fraction (14 μg/mL).

2.7. Seed exudates

Glycine max seeds were washed with 30% ethanol and arranged into Petri dishes (20 seeds per Petri dish) containing 6 mL of 100 mM sodium acetate buffer (pH 5.0). Seeds were allowed to imbibe in a controlled environment cabinet (28 °C and relative humidity 60%) for 20 h. After the first hour and then after every 5 h of imbibition, seeds were removed from the incubator and the volume contained in the Petri dishes was collected and measured. Seed exudates thus collected were submitted to protein quantification by the Bradford method (1976) and analysed for acid phosphatase and peroxidase activities as previously described.

3. Results

3.1. Isolation and antifungal in vitro activity of seed coat fraction

For isolation of *G. max* seed coat antifungal fractions, 0–90% ammonium sulphate precipitate was submitted to ion exchange chromatography on DEAE-Sepharose (Fig. 1). Adsorbed samples were desorbed by 0.25 and 0.5 M NaCl (DII and DIII fractions, respectively). All chromatographic fractions and the LMW fraction were tested against phytopathogenic fungi. Assays for fungal growth inhibition activity showed that DIII was the only fraction to inhibit fungal growth at each time point after 24 h for both *F. lateritium* (Fig. 2A) and *F. oxysporum* (Fig. 2B). This fraction inhibited about 42.6% and 71.7% of the growth of *F. lateritium* at 42 and 48 h, respectively. Inhibition of *F. oxysporum* at 42 and 48 h was similar (40.5% and 65.8%, respectively). The DII fraction showed a lesser but still statistically significant inhibition action against *F. lateritium* at 36 h (Fig. 2A) and against *F. oxysporum* at 36, 42 and 48 h (Fig. 2B).

3.2. Characterization of the antifungal fraction proteins

Analysis of the DIII fraction by SDS–PAGE showed the presence of protein bands with molecular masses between 66 and 15 kDa

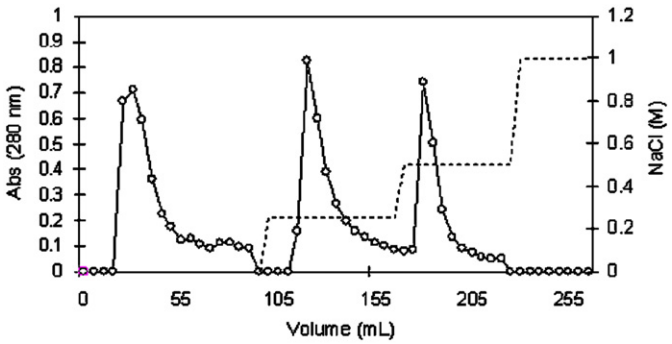

Fig. 1. Chromatogram on DEAE-Sepharose of *Glycine max* seed coat proteins previously precipitated by ammonium sulphate (0–90% saturation). The column was equilibrated and chromatography was developed with 50 mM potassium phosphate buffer, pH 7.6 (DI fraction). Adsorbed proteins were desorbed by 0.25 and 0.5 M NaCl (DII and DIII fraction, respectively).

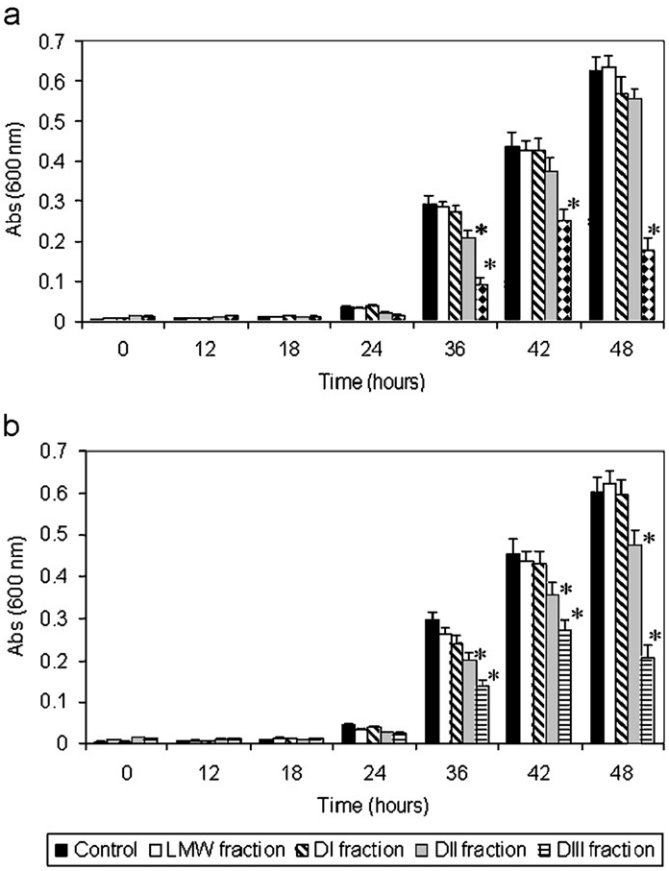

Fig. 2. Effects of low molecular weight fraction (LMW fraction) and DEAE-Sepharose chromatographic fractions (DI: non-retained fraction, DII: fraction desorbed by 0.25 M NaCl, DIII: fraction desorbed by 0.5 M NaCl) on the growth of (A) *Fusarium lateritium* and (B) *Fusarium oxysporum*. Experiments were run in triplicate and the data shown are the averages of these results. Means denoted with an asterisk (*) indicate a significant difference from the controls ($P<0.05$, Student's *t*-test).

(Fig. 3A). A protein with Mr ≅ 24 kDa was identified with an amino acid sequence similar to an acid phosphatase from *Synechocystis* sp. (Fig. 3B). A band with acid phosphatase activity was found among DIII fraction proteins separated by native-PAGE (Fig. 3C). The DIII fraction had 0.17 mAU/μg P (milliunit of activity per microgram of protein), as determined by *in vitro* measurement of the acid phosphatase. This enzyme activity showed an optimal activity time at 25 min, optimal temperature at 37 and 50 °C, optimal pH at 4.0 and showed high activity at 50 °C (Fig. 3D).

Vicilin-like proteins were also present in the soybean seed coat (Fig. 4A). ELISA assays showed that vicilin-like proteins were present in flour from the seed coat at 5.4 ng/μg and were four times more concentrated in the DIII fraction (20.4 ng/μg, Fig. 4B). A protein band in the DIII fraction indicated peroxidase activity when using DAB as substrate (Fig. 5A) and the presence of peroxidase was confirmed by the detection of *in vitro* activity with *p*-NPP, which revealed a peroxidase activity of 1000 mAU/μg P. Trypsin inhibitor and chitinase activities were not detected in the DIII fraction by the methods used. Acid phosphatase and peroxidase activities were detected in seed exudates, with higher levels present at 20 h (Fig. 5B). The acid phosphatase activity was detected in the first hour of imbibition and it continuously increased until 20 h of imbibition, when the amount of acid phosphatase exuded per seed reached 0.7 AU (Fig. 5B). The peroxidase activity was detected only at 20 h of seed imbibition and the exuded amount was ca. 2 AU/seed (Fig. 5B).

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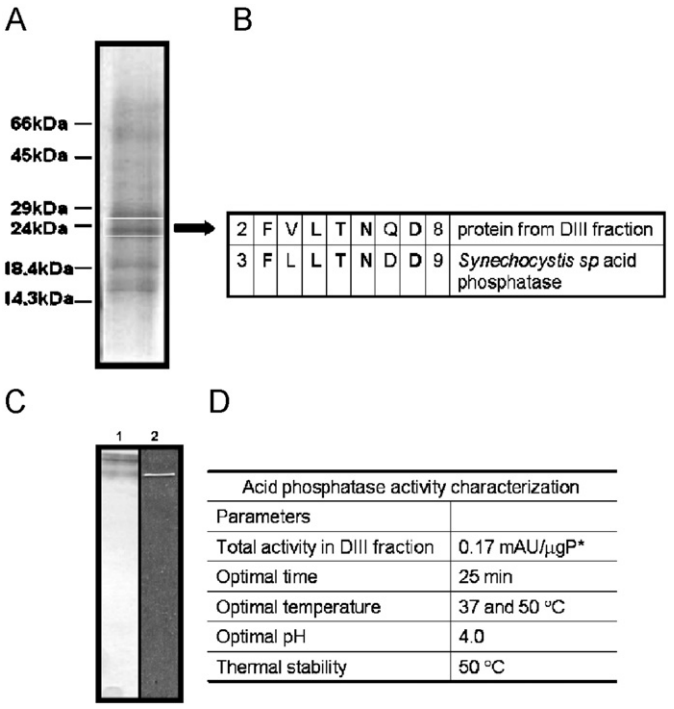

Acid phosphatase activity characterization	
Parameters	
Total activity in DIII fraction	0.17 mAU/μgP*
Optimal time	25 min
Optimal temperature	37 and 50 °C
Optimal pH	4.0
Thermal stability	50 °C

Fig. 3. (A) Visualization of proteins from seed coat DEAE-Sepharose chromatography DIII fraction separated by SDS-polyacrylamide gel electrophoresis (15%), (B) N-terminal amino acid sequence of Mr ≅ 24 kDa protein from the DIII fraction (the arrow indicates the protein band submitted to amino acid sequence determination), (C) visualization of proteins from the DIII fraction by native-polyacrylamide gel electrophoresis and acid phosphatase activity from the DIII fraction, and (D) characterization of the DIII fraction acid phosphatase activity. One unit of acid phosphatase activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol *p*-NPP, or the formation of 1 μmol inorganic phosphate, per minute.

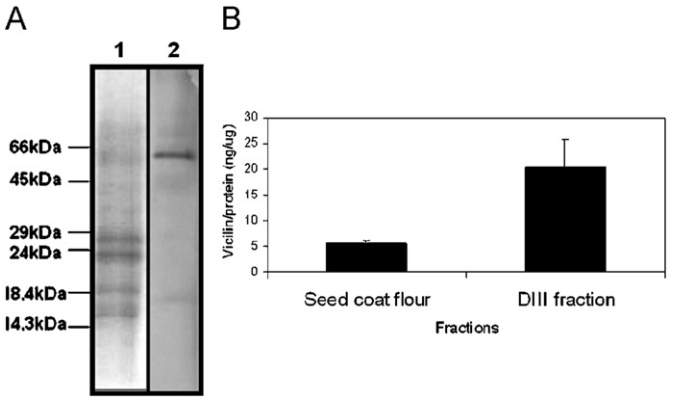

Fig. 4. (A) Visualization of proteins from the DIII fraction separated by SDS-polyacrylamide gel electrophoresis (15%) (lane 1) and vicilin detection by Western blotting (lane 2) and (B) vicilin levels in *Glycine max* seed coat flour and DIII fraction, measured by ELISA assay using an anti-*Vigna unguiculata* vicilin antibody. Experiments were run in triplicate and the percentages shown are the averages of these results.

These experiments showed that proteins were exuded during seed imbibition, the maximum level of exuded proteins (120 μg/seed) detected being at 20 h after the start of imbibition (Fig. 5C). The exuded acid phosphatase and peroxidase activities at this specific time were ca. 5.8 and 0.016 AU/μg P, respectively.

4. Discussion

The development of novel antifungal agents (natural or synthetic) is a reasonable global agricultural demand for the deterrence of infections caused by phytopathogenic fungi. The understanding of the defense mechanisms used by plants to repel pests and pathogens could be used in breeding programs for host–plant resistance. With this aim, researches are oriented more and more towards the search for molecules that possess a selective action against fungi, without being toxic to other organisms or to ecosystems. Although an enormous number of toxic proteins and peptides found in seed cotyledons have been related to the resistance of some seeds against pathogens, almost no work has considered the toxicity of the seed coat as an important factor for such resistance, even though this tissue is the first natural barrier encountered by pests of stored seeds.

In this work, we have shown that the *G. max* seed coat has compounds which are toxic to the fungi *F. lateritium* and *F. oxysporum* (Fig. 2A and B, respectively). These compounds were all found in an acidic fraction retained by a DEAE-Sepharose matrix. Analysis of this DIII fraction by SDS–PAGE showed the presence of a 24 kDa acid phosphatase protein (Fig. 3B) and this enzyme activity had optimal temperatures at 37 and 50 °C, optimal pH at 4.0 and showed high activity at 50 °C (Fig. 3D), which is higher than that described for *G. max* seed cotyledons acid phosphatase (Ferreira et al., 1998). The existence of two optimal temperatures of activity may indicate the existence of isoenzymes in this fraction. In the DIII fraction, acid phosphatase activity was at a level of 0.17 mAU/μg P. Considering the 40 μg of this DIII fraction used in the fungal assay (which corresponds to 6.8 mAU), we conclude that 6.8 mAU of the enzyme is enough to inhibit by 71.7% and 65.8% the growth of *F. lateritium* and *F. oxysporum*, respectively, at 48 h. Acid phosphatases are a group of enzymes that catalyse the hydrolysis of phosphate monoesters. In plants, acid phosphatases have been characterized in roots, tubers, bulbs, seed cotyledons, the aleurone layer, leaves, maize scutellum and suspension cells (Zhang and McManus, 2000) and are involved in phosphate metabolism and appear to function in response to phosphate deficiency, salt stress (Pan, 1987) and water deficit (Barrett-Lennard et al., 1982). Many other roles have been ascribed to acid phosphatases in plants, including participation in signal transduction and regulation of metabolism by protein dephosphorylation (Struglics et al., 2000).

Seed storage vicilin-like proteins were also present in the antifungal fraction from the soybean seed coat (Fig. 4A and B). Inhibitory activity against fungi has previously been reported for vicilin-like proteins. Chung et al. (1997) have shown that the filamentous fungi *Botrytis cinerea* (De Bary) Whetzel, *Alternaria brassicicola* (Schwein.) Wiltshire, *Chalara elegans* Nag Raj & Kendrick and *F. oxysporum* had their growth inhibited by vicilin-related proteins from *Gossypium hirsutum* L. seeds. Vicilin 7S storage proteins isolated from different legume seeds were shown to inhibit yeast growth and glucose stimulated acidification of the medium by yeast cells (Gomes et al., 1998). These authors have also observed that vicilins bind to yeast cells, possibly through vicilin association with chitin-containing structures of yeast cells. They suggest that such association could result in the observed inhibition of H^+ pumping, cell growth and spore formation (Gomes et al., 1998).

Other soybean seed coat abundant proteins have previously been identified, among these are a peroxidase enzyme of 41 kDa (Buttery and Buzzel, 1968; Gijzen, 1997), a 32 kDa class I chitinase (Gijzen et al., 2001), a 21 kDa trypsin inhibitor (Kunitz, 1945; Koide and Ikenaka, 1973) and an 8 kDa hydrophobic protein (Gijzen et al., 1999). For this reason, the presence of some of these known proteins was investigated in the DIII fraction. A protein band in this fraction showed peroxidase activity (Fig. 5A). Peroxidases have

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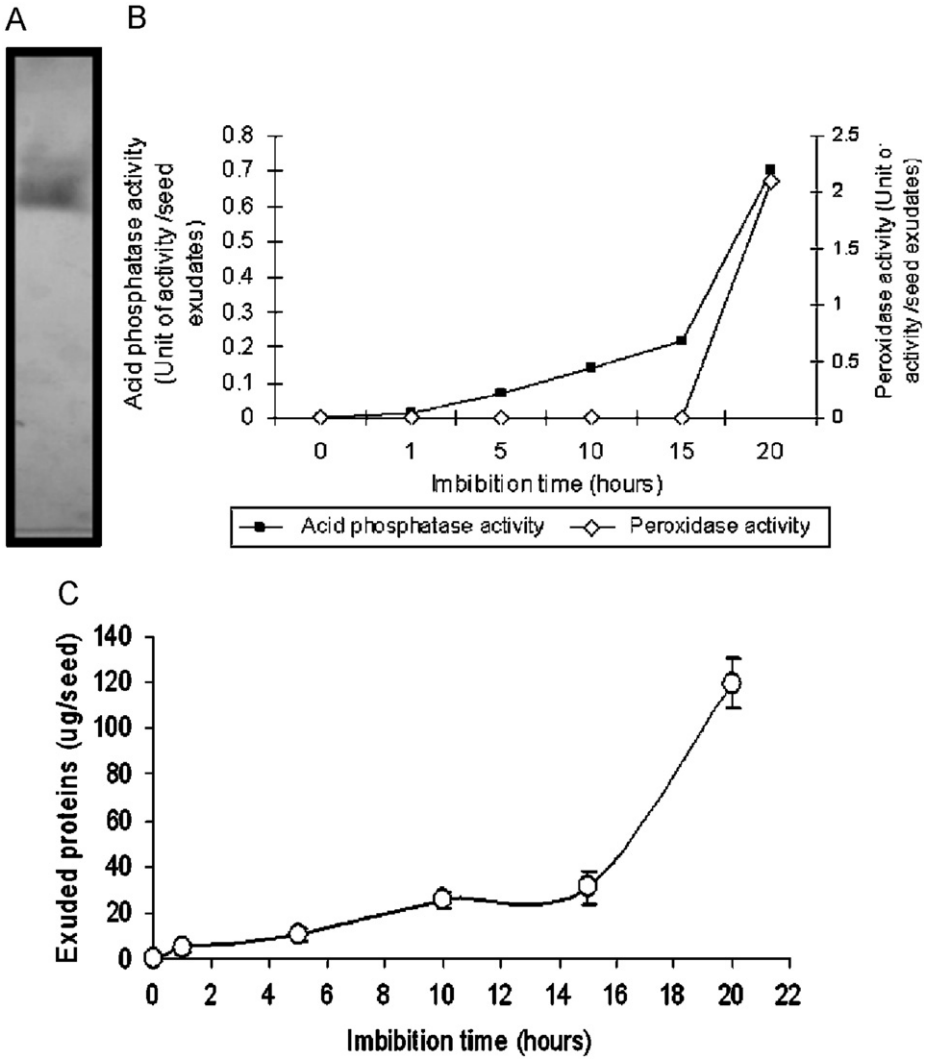

Fig. 5. (A) Visualization of peroxidase activity in nitrocellulose membrane from DIII proteins separated by native-polyacrylamide gel electrophoresis (15%), (B) acid phosphatase and peroxidase exudation profile from *Glycine max* seeds over 20 h of imbibition (unit of activity/seed), and (C) protein quantification (μg/seed) over 20 h of imbibition. Each point represents the mean of triplicate determinations. One unit of acid phosphatase activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol *p*-NPP, or the formation of 1 μmol inorganic phosphate, per minute. One unit of peroxidase activity is defined as the amount of enzyme that catalyses an absorbance increase of 0.01 at 470 nm/min.

been implicated in a variety of physiological processes in plants, such as lignin biosynthesis, extensin polymerization, auxin metabolism, wound healing and disease resistance (Siegel, 1993). Soybean seed coat peroxidases (SBPs) were shown to be very stable at high temperatures, extremes of pH and in organic solvents (Nissum et al., 2001). The mature protein showed higher than 70% amino acid sequence identity to peroxidases from other legumes recruited in various defense response processes (Henriksen et al., 2001). Examples of such plant defense related to peroxidases are found in Hammerschmidt et al. (1982) and El-Turk et al. (1996). Hammerschmidt et al. (1982) showed the association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium* (Pass.) Ell. & Halst. El-Turk et al. (1996) reported the nucleotide sequences of four pathogen-induced alfalfa peroxidase-encoding cDNAs.

Gijzen et al. (2001) showed that a 32 kDa class I chitinase is an abundant protein in soluble extracts from soybean seed coat tissues. However, chitinase activity was not detected in the DIII fraction by the methods we used. Trypsin inhibitor activity was also not detected in this more closely analysed DIII fraction, although a 21 kDa trypsin inhibitor has also been previously detected in soybean seed coat (Kunitz, 1945; Koide and Ikenaka, 1973).

A protective role for proteins during seed germination has been proposed due to their exudation. Based upon this idea, we analysed the profile of exudation of imbibed *G. max* seeds and the possible presence of proteins in these exudates. The results showed that acid phosphatase and peroxidase activities were detected in seed exudates. In these experiments, we observed an acid phosphatase exudation of 0.7 AU/seed (or 700 mAU); thus, each seed exuded an amount ca. 103 times higher than the estimated amount of 6.8 mAU, able to cause significant fungal inhibition. These results reinforce the idea that these proteins could be important for the protection of seeds against pathogens during the germination period. Our results suggest that soybean seed coat proteins can participate in the constitutive defense mechanisms of these seeds against fungal attack.

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